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High throughput PCR detection of *Xylella fastidiosa* directly from almond tissues

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Abstract

Xylella fastidiosa, the causal agent of almond leaf scorch disease (ALSD), is currently re-emerging as a serious concern in California. Efficient pathogen detection is critical for ALSD epidemiological studies, particularly when a large sample size is involved. We here report a PCR procedure to detect *X. fastidiosa* directly from infected almond tissue without the laborious DNA extraction. Plant samples were prepared by freeze-drying and pulverized. Appropriate dilutions of the pulverized freeze-dried tissue (PFT) were determined to minimize the effect of enzyme inhibitors from plant tissue and retain PCR detection of *X. fastdiosa* cells at a single digit number level. This PFT-PCR procedure was evaluated by comparing to the *in vitro* cultivation method using 102 symptomatic samples and resulted in a predictive value of 90.8%. PFT-PCR was further applied to monitor the seasonal occurrence of *X. fastidiosa* from four selected almond trees in two orchards in 2005. The results matched with those of the cultivation method at 92.3%. Considering the simplicity and reliability, we conclude that PFT-PCR is a valuable option for high throughput rapid detection of *X. fastidiosa*.
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Keywords: Almond leaf scorch; Freeze-drying; High throughput detection; Pathogen detection; PFT-PCR; *Xylella fastidiosa*

1. Introduction

Almond leaf scorch disease (ALSD) is currently re-emerging in the San Joaquin Valley (SJV) of California, potentially threatening the production of this highly valued crop. The epidemiology of ALSD is poorly understood and the potential economic effects of the disease are unknown. ALSD is caused by *Xylella fastidiosa*, a nutritionally fastidious bacterium (Wells et al., 1987). Symptoms of ALSD are typically leaf marginal necrosis or leaf scorching. These symptoms are not a specific indication of ALSD and can be easily confused with salt toxicity and mineral deficiency commonly occurring in SJV (Mircetich et al., 1976). The primary diagnosis of ALSD relies on pathogen detection.

Isolation by pathogen cultivation *in vitro* is the most definitive and direct for pathogen detection and identification because whole bacterial cells and their biochemical and physiological properties are observed. However, cultivation of *X. fastidiosa* in the laboratory is time consuming, ranging from 3 to 20 days, and labor intensive, particularly when a large number of samples are involved. Serological methods target the unique properties of bacterial cell surface. Among them, enzyme-linked immunosorbent assay (ELISA) is commonly used and has a high throughput capacity (Sherald & Lei, 1991) because of the simplicity in sample preparation and the use of the 96-well plate format. However, production of antiserum is a complicated process and cross reactions between different *X. fastidiosa* strains remains to be a problem with the currently available antiserum.

PCR detection targets the variation of bacterial genomic DNA. The available whole genome sequences of *X. fastidiosa* strains (Bhattacharyya et al., 2002; Simpson et al., 2000; Van Sluys et al., 2003) make it feasible to design PCR primers at various levels of

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specificity. Several specific PCR primer sets are currently available for *X. fastidiosa* detection including the most thoroughly tested RST31/33 primer set (Minsavage et al., 1994), derived from the RNA polymerase genomic locus, and those derived from 16S rRNA gene (Chen et al., 2005), an important taxonomical character for the description of *X. fastidiosa* (Wells et al., 1987). In contrary, sample preparation methods have been subjected to much less vigorous evaluation.

The most common PCR detection procedures for *X. fastidiosa* involve sample extraction to generate template DNA. However, the current DNA extraction procedure is very time consuming, severely reducing the high throughput capacity of a PCR technique. Efforts were made to simplify or omit the DNA extraction procedure by using plant sap for PCR DNA template, but the results were inconsistent (Banks et al., 1999; Minsavage et al., 1994). In this study, we developed and evaluated a PCR procedure to detect *X. fastidiosa* from pulverized freeze-dried tissues (PFT-PCR, namely) without the need for DNA extraction. Our hypothesis was that bacterial DNA would be released into detection buffer if the plant tissue was sufficiently pulverized and thereby provided adequate quantity and quality of template DNA for PCR analysis.

2. Materials and methods

2.1. Sample collection and preparation

All almond leave samples were collected from commercial orchards in SJV of California. The leaf sample was first used for bacterial isolation, and, then, the same piece of sample was used for PFT-PCR. Two evaluation procedures were used. The first procedure was to test the predictive capacity PFT-PCR to the bacterial cultivation method, and the second procedure was to test if PFT-PCR could be used to monitor the population dynamic of *X. fastidiosa* in selected trees in two orchards during a growing season.

For the first evaluation, samples were collected from 102 almond trees in an orchard in Fresno County, California, in September, 2004. Briefly, small branches showing leaf scorching symptoms were excised, placed in labeled plastic bags, and transported in an ice cooler to the SJV Agricultural Sciences Center, Parlier, California. Upon arrival, samples were stored at 4 °C and processed within 24 h. One symptomatic leaf was selected to represent one tree. For the second evaluation, two almond orchards in Fresno County, California, were selected in 2005 based on the presence of ALSD in 2004. One previously known ALSD tree and one tree which did not show any leaf scorching symptoms were selected from each almond orchard. The branching patterns of each tree were mapped. Leaf samples were collected from labeled scaffolds in May and June when no leaf scorching symptoms were seen and in September when symptoms were obvious. Sample collection and processing were identical to that of the first evaluation.

2.2. Bacterial isolation and cultivation

The previously described procedure (Chen et al., 2005) was followed. Briefly, petioles of approximate 2 cm length were separated from leaves and surface sterilized in 10% sodium hypochlorite for two minutes followed by three successive rinses in sterile, distilled water. An incision was made in the center of the petiole. Xylem sap was expressed aseptically using a pair of flame-sterilized, needle-nose pliers onto the sterile surface of a Petri dish. A drop of PW broth (Davis et al., 1981) was immediately added and mixed with the sap. One loopful of the sap mixture was then streaked onto PW medium solidified by Gelrite (Sigma-Aldrich, Inc. St. Louis, Mo) and incubated at 28 °C. The appearance of opalescent colonies was monitored using a binocular microscope for up to 40 days. Candidate isolates were transferred onto new PW-G and confirmed as *X. fastidiosa* by PCR using the RST 31/33 primers (Minsavage et al., 1994) and 16S rDNA primers (Chen et al., 2005).

2.3. Freeze-drying and sample pulverization

Almond leaves or petiole leftovers from isolation experiment were placed in a labeled paper envelope and freeze-dried in the Freezone 2.5 Freeze Dry System (Labconco Corp., Kansas City, Mo) overnight. Almond petioles were sufficiently dried overnight (>12 h) in the freeze-dryer following the manufacturers' recommendation (Temperature <−40 °C; and Vacuum $<1.33\times10^{-3}$ mBAR). The dried samples were used immediately or stored at 4 °C in plastic bags. Individual freeze-dried leaf petioles with approximate length of 2 cm were placed into 2 ml microtubes with sterile ceramic beads and pulverized to a fine powder in a Fast-Prep machine (FP120, Qbiogene, Inc. Carlsbad, CA) for 20 s. The dried tissues were easily pulverized by FastPrep machine within 20 s. The pulverized freeze-dried tissue or PFT was suspended in 500 µl of TE buffer and used for PCR after dilution. PFT particle sizes were measured microscopically using a micrometer.

2.4. PCR procedure

PCR reaction (25 µl) was carried out using the TaKaRa TaqTM (Hot Start Version) kit (Takara Bio Inc., Seta 3-4-1, Otsu, Shiga, 520-2193, Japan). The reaction mixture contained: 2.5 µl of 10×DNA polymerase buffer, 2.5 µl of dNTPs (2.5 mM of each dNTP), 0.5 µl of each of the 10 µM forward and reverse primers, 1 µl of diluted petiole suspension, 0.2 µl of *Taq* DNA polymerase (5 U/µl) and 18.3 µl of H_2O. The multiplex PCR procedure (Chen et al., 2005) was used for *X. fastidiosa* detection. Briefly, primers Teme150fc (5′ tctaccttat cgtgggggac 3′) and Teme454rg (5′ aacaactagg tattaaccaa ttgcc 3′) specific to G-genotype and primers Dixon454fa (5′cctttgttg gggaagaaaa 3′) and Dixon1261rg (5′ tagctcaccc tcgcgagatc 3′) specific to A-genotype, were used for PCR amplification in an MJ Research Tetrad II DNA engine with an initial denaturing at 96 °C for 10 min, followed by 30 cycles consisting of: denaturing at 96 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. The amplification products were stored at 4 °C. The amplified DNAs were resolved through 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. To further confirm that *X. fastidiosa* DNA was amplified, PCR amplicons were directly sequenced using a 3130xl Genetic Analyzer (Applied Biosystem, Foster City, California, USA).

2.5. Determination of PFT dilutions for PCR

From a 10 days old *X. fastidiosa* strain Temecula-1 culture on PWG, a loop of culture was collected and suspended in 100 µl of sterile water. A series of 10 fold dilution was made and bacterial concentration in colony forming unit (CFU) was determined on PWG with three replicates after 10 day incubation. Water suspensions containing 1.4×10^5 and 9×10^3 CFU/ml were used to make a serial dilution of PFT from a healthy almond tree (petiole). For the convenience of discussion, a CFU was interpreted as a *X. fastidiosa* cell. One µl of PFT-bacterial suspension, representing either 137 or 9 cells, was used for PCR.

2.6. Data analyses

Results from *in vitro* cultivation were considered as a "gold standard" and used to compare the PFT-PCR results. There are four possible outcomes: true positive, false positive, true negative and false negative (Table 1). Two parameters were introduced for the first PFT-PCR evaluation: a predictive value of the positive test and a predictive value of the negative test. These parameters were adopted from medical epidemiological studies (Kelsey et al., 1996) and defined in Table 1. For the second evaluation (population dynamic experiment), results of symptom expression, PFT-PCR detection, and cultivation were compared and correlation percentages were calculated.

3. Results

In a pilot experiment, PCR using 1 µl of the 1:500 diluted symptomatic tissue suspensions and the 1:10 dilution (a total of 1:5000) dilution showed inconsistent DNA amplification, presumably due to the presence of polymerase inhibitor(s). At the dilution of 1:100 (a total of 1:50,000 dilution), a satisfactory detection rate (10/10) was achieved. This was also demonstrated in the spike experiment (Fig. 1). With the bacterial cell count at 9, *X. fastidiosa* was detected at the 1:100 and thereafter dilutions of the 1:500 PFT suspension. At a higher bacterial titer (137 cells), lower dilutions were needed. Microscopic examination of the PFT particles showed that the size ranged from 1.5 to 6.6 µm with a mean of 3.0 µm ($n=20$) and the weight ranged from 4 to 16 mg with a mean weight of 10 mg ($n=10$) for an approximate petiole length of 2 cm. One µl of the 1:50,000 diluted suspensions contained about 0.2 µg of host tissue.

Table 1
Evaluation of PCR detection of *Xylella fastidiosa* in pulverized freeze-dried almond petiole tissue comparing to *in vitro* cultivation method

		Pathogen cultivation	
		Positive = 85	Negative = 17
PCR	Positive = 65	59 (*a*, True +)	6 (*b*, False +)
Detection	Negative = 37	26 (*c*, False −)	11 (*d*, True −)

Predictive value of a Positive test $= a/(a+b) = 59/(59+6) = 90.8\%$
Predictive value of a Negative test $= d/(c+d) = 11/(26+11) = 29.7\%$

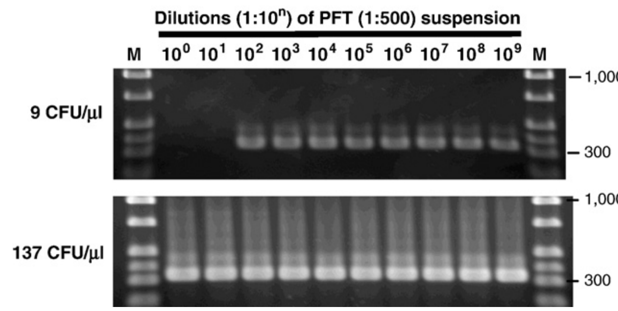

Fig. 1. Evaluation of dilution effects on PCR detection of *Xylella fastitidiosa* from pulverized freeze-dried tissue (PFT) of almond. *X. fastidiosa* strain Temecula-1 was spiked into PFT suspension of a healthy almond tree. At 9 CFU/µl, *X. fastidiosa* was detected at the 1:50,000 (1:100 of 1:500) and thereafter dilutions. At 137 CFU/µl, *X. fastidiosa* was detected directly from the 1:500 dilution. M, DNA markers with their sizes in bp indicated by the numbers on the right.

All candidate bacterial colonies from isolation experiments were confirmed to be *X. fastdisoa* by PCR using primers RST31/33 (Minsavage et al., 1994) and 16S rDNA primers (Chen et al., 2005), both primer sets were specific to *X. fastidiosa*. All *X. fastidiosa* strains derived from this study belonged to G-genotype, as indicated by the presence of the ~350 bp amplicons identical to those shown in Fig. 1. Comparison of 8 DNA sequences from PFT-PCR amplicons further showed that *X. fastidiosa* DNA was amplified. A representative sequence was deposited in GenBank with an accession number of EU413889.

In the first (PFT-PCR vs. bacterial cultivation) evaluation, 85 of 102 samples (83.3%) were positive based on pathogen isolation (Table 1). PFT-PCR detected 65 (63.7%) positive samples. Among the *X. fastidiosa* positive samples, 59 were shared by both methods. The true positive rate of PFT-PCR was 69.4% (Table 1). Only 6 samples were PFT-PCR positive but isolation negative. These were considered to be false positive. The predictive value of PFT-PCR for a positive test was, therefore, calculated to be 90.8%. On the other hand, 11 *X. fastidiosa*-negative samples by isolation and PFT-PCR were defined as true negative (64.7%). Twenty-six PFT-PCR negative samples were in fact isolation positive and were considered to be false negative. The predictive value of the PFT-PCR method for a negative test was 29.7% (Table 1).

The results of population dynamics of *X. fastidiosa* in four almond trees during 2005 are presented in Table 2. The trees in the two orchards did not show ALSD symptoms until July. With one exception, all samples collected in May were negative by both PFT-PCR and pathogen cultivation. *X. fastidiosa* was isolated from scaffold B in Tree 2. In late June, the pathogen was detected by both PFT-PCR and cultivation method from the two trees known to be *X. fastidiosa*-positive in 2005 by leaf scorch symptoms and PCR test. In September, tree 2 (non-symptomatic in 2004) of orchard 2 also showed typical leaf scorching symptoms. All symptomatic trees were confirmed to be *X. fastidiosa*-positive by both PFT-PCR and pathogen cultivation. Tree 2 of orchard 1 remained non-symptomatic and negative with both PFT-PCR and pathogen cultivation. With the total of 52 samples, the detection results of PFT-PCR and

Table 2
Seasonal detection of *Xylella fastidiosa* in samples from two almond orchards in Fresno County of California in 2005

Tree	Branch	5/24/06			6/30/06			9/8/06		
		CUL	PCR	SYM	CUL	PCR	SYM	CUL	PCR	SYM
Orchard 1										
Tree 1	A	−	−	−	+	G	−	+, +	G, G	+
	B	−	−	−	+	G	−	+, +	G, G	+
	C	−	−	−	+	G	−	+, +	G, G	+
	D	−	−	−	−	G	−	+, +	G, G	+
Tree 2	A	−	−	−	−	−	−	−, −	−, −	−
	B	+	−	−	−	−	−	−, −	−, −	−
	C	−	−	−	−	−	−	−, −	−, −	−
Orchard 2										
Tree 1	A	−	−	−	+	G	−	+, +	G, G	+
	B	−	−	−	+	G	−	+, +	G, G	+
	C	−	−	−	+	G	−	+, +	G, G	+
Tree 2	A	−	−	−	−	−	−	−, +	G, −	+
	B	−	−	−	−	−	−	+, −	G, −	+
	C	−	−	−	−	−	−	+, +	G, G	+
	H_2O		−			−			−	
X. fastidiosa Dixon			A			A			A	
X. fastidiosa Temecula-1			G			G			G	

Note: CUL = Bacterial isolation method; PCR = Polymerase chain reaction from pulverized freeze-dried tissue. "+" and "−" represent a positive and a negative result, respectively. "G", denotes a G-genotype (348 bp amplicon) and "A", denotes an A-genotype (847 bp amplicon). SYM = Symptom of leaf scorching.

isolation method agreed in 48 (23 positive and 25 negative), or in other words a 92.3% match.

4. Discussion

Freeze-drying is a common protocol for DNA extraction from plant tissues (Gawel & Jarret 1991; Geuna et al., 2000; Stoger & Ruppitsch 2004). DNA extracted from Freeze-dried plant tissue has been successfully used to detect bacterial pathogens (Chen et al., 1992; Stoger & Ruppitsch, 2004). With the FastPrep machine, 24 samples can be pulverized simultaneously. Instruments that can simultaneously pulverized 96 samples are also commercially available for higher throughput sample preparation. The use of freeze-dried plant tissue offers three advantages: 1) freeze-dried tissues are mechanically easily pulverized into small particles; 2) there are no tissue oxidation and enzymatic degradation of sample DNA during the pulverization process, because the sample tissues are dehydrated; and 3) freeze-dried tissues could be stored at 4 °C or even room temperature for an extended period of time. Samples collected at different times can be accumulated and processed concurrently.

In infected trees, *X. fastidiosa* resides in xylem vessels. Free DNA in solution is a precondition for PCR. In this study, the labor intensive DNA extraction step was replaced by a simple suspension dilution. The pulverization procedure macerated the almond petiole tissues into small particles and dramatically increased the host tissue surface area. We calculated that, for a $20 \times 1 \times 1$ mm^3 petiole, the surface area is about 80 mm^2. After freeze-drying and pulverization of the tissues, the total surface area increased to 40,000 mm^2 with the particle size of 3.0 μm (or $3 \times 3 \times 3$ μm^3). The extensive exposure of internal surface significantly increases the probability of releasing more *X. fastidiosa* cells/DNAs from the host tissues into the detection buffer medium and serves as the source of DNA template for PCR analysis.

However, as bacterial cells/DNAs were liberated, compounds inhibiting DNA polymerase could also be released from host tissue as evidenced by the inconsistent PFT-PCR results using 1:500 dilutions as template. Dilution of the PFT reduces the concentration of enzyme inhibitors but would also reduce the concentration of DNA template beyond the detection range of PCR. The best dilution for a sample depends on the bacterial titer which is highly variable and difficult to predict. We determined that the 1:50,000 (1:500 followed by 1:100) dilution is sufficient to minimize the effect of enzyme inhibitors from almond tissue and detect *X. fastidiosa* cells at a single digit number level. In an experiment in which pure *X. fastidiosa* DNA (40 ng) was added to the PFT suspension from a healthy plant, we estimated that the sensitivity of *X. fastidiosa* detection by PFT-PCR was one log unit less than that of DNA in pure water (data not shown).

Since PFT-PCR and pathogen isolation were performed simultaneously from the same petiole, we were able to evaluate the predictive capacity of PFT-PCR for the "gold standard" cultivation method. As shown in Table 1, PFT-PCR has a high predictive value (90.8%) for the true positive samples, but a low predictive value for a true negative result (29.7%). These values suggest that the power of PFT-PCR is in inferring a positive result rather than a negative result. That is, a PFT-PCR positive result suggests, with high confidence, the presence of *X. fastidiosa* in the sample. However, a PFT-PCR negative result does not appear to be a reliable indication of the absence of *X. fastidiosa* in the sample.

As shown in Table 1, the pathogen cultivation method detected 20 more *X. fastidiosa* positive samples than PFT-PCR, suggesting that pathogen cultivation method is more reliable. It is still a routine test in our laboratory when sample size is small (<30) and when time is not a constraint. It should be noted that in this evaluation, *X. fastidiosa* were isolated from fully symptomatic leaves in September. Detection accuracy or reliability from asymptomatic samples is difficult to evaluate. Asymptomatic samples could be free of pathogen, low in titer and/or distributed unevenly, leading to a high sampling error as shown by the difference between PFT-PCR and isolation method (Table 2). PFT-PCR is advantageous in that it is more rapid and much less labor intensive than *X. fastidiosa* isolation method. Therefore, PFT-PCR is useful for *X. fastidiosa* screening from a large number of samples.

In summary, PFT-PCR is better suited for high throughput pathogen detection involving a large quantity of samples, such as epidemiological studies and germplasm resistance evaluation because no DNA extraction process is needed. Combined with leaf scorching symptoms, PFT-PCR has a 90.8% predicting value for the “gold standard” isolation method. For a population dynamic study, a 92.3% match between the two methods can be expected. The slight decrease in detection sensitivity of PFT-PCR is justified by its throughput capacity. The advantage of PFT-PCR will be even more obvious when a 96-well format similar to that of ELISA is developed. The PFT-PCR procedure described in this paper also has potential to be adapted to other disease systems. In our laboratory, PFT-PCR is also successfully used for grape for Pierce’s disease (*X. fastidiosa*) diagnosis.

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References

Banks, D., Albibi, R., Chen, J., Lamikanra, O., Jarret, R.L., Smith, B.J., 1999. Specific detection of *Xylella fastidiosa* Pierce’s disease strains. Curr. Microbiol. 39, 85–88.

Bhattacharyya, A., Stilwagen, S., Reznik, G., Feil, H., Feil, W.S., Anderson, I., Bernal, A., D’Souza, M., Ivanova, N., Kapatral, V., Larsen, N., Los, T., Lykidis, A., Selkov Jr., E., Walunas, T.L., Purcell, A., Edwards, R.A., Hawkins, T., Haselkorn, R., Overbeek, R., Kyrpides, N.C., Predki, P.F., 2002. Draft sequencing and comparative genomics of *Xylella fastidiosa* strains reveal novel biological insights. Genome Res. 12, 1556–1563.

Chen, J., Chang, C.J., Jarret, R.L., 1992. DNA probes as molecular markers to monitor the seasonal occurrence of walnut witches’-broom mycoplasmalike organism. Plant Dis. 76, 1116–1119.

Chen, J., Groves, E.L., Civerolo, M., Viveres, M., Freeman, M., Zheng, Y., 2005. Two *Xylella fastidiosa* genotypes associated with almond leaf scorch disease on the same location in California. Phytopathology 95, 708–714.

Davis, M.J., French, W.J., Schaad, N.W., 1981. Axenic culture of the bacteria associated with phony disease of peach and plum leaf scald. Curr. Microbiol. 6, 309–314.

Gawel, J.J., Jarret, R.L., 1991. Chloroplast DNA restriction fragment length polymorphisms (RFLPs) in *Musa* species. Theor. Appl. Genet. 81, 783–786.

Geuna, F., Hartings, H., Scienza, A., 2000. Plant DNA extraction based on grinding by reciprocal shaking of dried tissue. Anal. Biochem. 278, 228–230.

Kelsey, J.L., Whittemore, A.S., Evans, A.S., Thompson, W.D., 1996. Methods in Observational Epidemiology. Oxford University Press, Oxford.

Minsavage, G.V., Thompson, C.M., Hopkins, D.L., Leite, R.M.V.B.C., Stall, R.E., 1994. Development of a polymerase chain reaction protocol for detection of *Xylella fastidiosa* in plant tissue. Phytopathology 84, 456–461.

Mircetich, S.M., Lowe, S.K., Moller, W.J., Nyland, G., 1976. Etiology of almond leaf scorch disease and transmission of the causal agent. Phytopathology 66, 17–24.

Sherald, J.L., Lei, J.D., 1991. Evaluation of rapid ELISA test kit for detection of *Xylella fastidiosa* in landscape trees. Plant Dis. 75, 200–203.

Simpson, A.J.G., Reinach, F.C., Arruda, P., Abreu, F.A., Acencio, M., Alvarenga, R., Alves, L.M.C., Arya, J.E., Baia, G.S., Baptista, C.S., Barros, M.H., Bonaccorsi, E.D., Bordin, S., Bove, J.M., Briones, M.R.S., Bueno, M.R.P., Camargo, A.A., Camargo, L.E.A., Carraro, D.M., Carrer, H., Colauto, N.B., Colombo, C., Costa, F.F., Costa, M.C.R., Costaneto, C.M., Coutinho, L.L., Cristofani, M., DiasNeto, E., Docena, C., ElDorry, H., Facincani, A.P., Ferreira, A.J.S., Ferreira, V.C.A., Ferro, J.A., Fraga, J.S., Franca, S.C., Franco, M.C., Frohme, M., Furlan, L.R., Garnier, M., Goldman, G.H., Goldman, M.H.S., Gomes, S.L., Gruber, A., Ho, P.L., Hoheisel, J.D., Junqueira, M.L., Kemper, E.L., Kitajima, J.P., Krieger, J.E., Kuramae, E.E., Laigret, F., Lambias, M.R., Leite, L.C.C., Lemos, E.G.M., Lemos, M.V.F., Lopes, S.A., Lopes, C.R., Machado, J.A., Machado, M.A., Madeira, A.M.B.N., Madeira, H.M.F., Marino, C.L., Marques, M.V., Martins, E.A.L., Martins, E.M.F., Matsukuma, A.Y., Menck, C.F.M., Miracca, E.C., Miyaki, C.Y., MonteiroVitorello, C.B., Moon, D.H., Nagai, M.A., Nascimento, A.L.T.O., Netto, L.E.S., Nhani, A., Nobrega, F.G., Nunes, L.R., Oliveira, M.A., deOliveira, M.C., deOliveira, R.C., Palmieri, D.A., Paris, A., Peixoto, B.R., Pereira, G.A.G., Pereira, H.A., Pesquero, J.B., Quaggio, R.B., Roberto, P.G., Rodrigues, V., Rosa, A.J.D., deRosa, V.E., deSa, R.G., Santelli, R.V., Sawasaki, H.E., daSilva, A.C.R., daSilva, A.M., daSilva, F.R., Silva, W.A., daSilveira, J.F., Silvestri, M.L.Z., Siqueira, W.J., deSouza, A.A., deSouza, A.P., Terenzi, M.F., Truffi, D., Tsai, S.M., Tsuhako, M.H., Vallada, H., VanSluys, M.A., VerjovskiAlmeida, S., Vettore, A.L., Zago, M.A., Zatz, M., Meidanis, J., Setubal, J.C., 2000. The genome sequence of the plant pathogen *Xylella fastidiosa*. Nature 406, 151–157.

Stoger, A., Ruppitsch, W., 2004. A rapid and sensitive method for the detection of *Xanthomonas fragariae*, causal agent of angular leafspot disease in strawberry plants. J. Microbiol. Methods 58, 281–284.

Van Sluys, M.A., de Oliveira, M.C., Monteiro-Vitorello, C.B., Miyaki, C.Y., Furlan, L.R., Camargo, L.E., da Silva, A.C., Moon, D.H., Takita, M.A., Lemos, E.G., Machado, M.A., Ferro, M.I., da Silva, F.R., Goldman, M.H., Goldman, G.H., Lemos, M.V., El-Dorry, H., Tsai, S.M., Carrer, H., Carraro, D.M., de Oliveira, R.C., Nunes, L.R., Siqueira, W.J., Coutinho, L.L., Kimura, E.T., Ferro, E.S., Harakava, R., Kuramae, E.E., Marino, C.L., Giglioti, E., Abreu, I.L., Alves, L.M., do Amaral, A.M., Baia, G.S., Blanco, S.R., Brito, M.S., Cannavan, F.S., Celestino, A.V., da Cunha, A.F., Fenille, R.C., Ferro, J.A., Formighieri, E.F., Kishi, L.T., Leoni, S.G., Oliveira, A.R., Rosa Jr., V.E., Sassaki, F.T., Sena, J.A., de Souza, A.A., Truffi, D., Tsukumo, F., Yanai, G.M., Zaros, L.G., Civerolo, E.L., Simpson, A.J., Almeida Jr., N.F., Setubal, J.C., Kitajima, J.P., 2003. Comparative analyses of the complete genome sequences of Pierce’s disease and citrus variegated chlorosis strains of *Xylella fastidiosa*. J. Bacteriol. 185, 1018–1026.

Wells, J.M., Raju, B.C., Hung, H.-Y., Weisburg, W.G., Mandelco-Paul, L., Brenner, B.J., 1987. *Xylella fastidiosa* new-genus new-species gram-negative xylem-limited fastidious plant bacteria related to *Xanthamonas*-spp. Int. J. Syst. Bacteriol. 37, 136–143.